SOMITE GENESIS IN THE CHICK. II. ANALYSIS OF NUTRIENTS FROM YOLK [1]

RONALD C. FRASER

Department of Zoology and Entomology, University of Tennessee, Knoxville, Tennessee

A previous communication has disclosed that there are a number of components in the albumen of the avian egg which collectively are used by the embryo in the development of somites (Fraser, 1957). These components were indicated to be such small molecular forms as glucose, alanine and a heat-labile substance. Moreover, the protein moiety of the albumen may possibly be used to a limited extent as well in this function.

Because the yolk provides the natural environment of the early chick blastoderm, it seemed advisable to assay this portion of the egg for its nutritional value with respect to the formation of somites. Studies by Needham (1931) and Romanoff and Romanoff (1949) have indicated that the yolk of the avian egg is chemically complex, containing such diverse molecular species as proteins, phospholipids, coenzymes, amino acids, etc.

It has become apparent from many studies that the embryo does not use indiscriminately all of the materials made available to it at any one period during development. For example, the transitional nature of energy sources has been pointed out by Needham (1950). Fraser (1956) has shown that the early blastoderm will starve on a fat diet, although there is much of this food substance present in the unincubated egg.

With respect to the utilization of certain nutrients for specific morphogenetic events, there exists but little information. Wilde (1955) has revealed the use of various portions of the phenylalanine molecule by neural crest cells of the amphibian in melanogenesis. Similarly, the development of the heart and of the brain of the chick embryo have been shown to have different sugar requirements (Spratt, 1950). The present paper represents an attempt to assay the yolk of the chicken egg for its nutritional value, specifically in somite genesis. Other papers to follow will cover other facets of this morphogenetic event.

Materials and Methods

While a few of the eggs used in the present investigation were from Rhode Island Red chickens, most were from White Leghorn hens. Nutritionally, the two breeds appear equivalent, although the rate of development of the former is perceptibly slower during the earliest phases of embryogenesis. The eggs were stored at 18° C. until incubated, in all instances within a week after they had been layed. Eggs were incubated at 38.0° C., while explants were cultured at 37.8° C.

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General methods for the culture of the early chick blastoderm have been outlined previously (Spratt, 1947; Fraser, 1957). Definitive primitive streak (DPS) blastoderms were removed from the eggs following approximately 21 hours of incubation, and placed on semi-solid media. After camera lucida diagrams had been made of them, the embryos were incubated for a period of 22 hours, after which time diagrams were again made for the purpose of recording the extent of development. The embryos were then fixed on the gel with Gerhard's fixative and prepared for whole mounts. Delafield's hematoxylin was used as a stain. Greater accuracy could be obtained in counting somites in stained preparations. Approximately six hundred explants were cultured in the course of this investigation.

Preparation of Media

The reader is referred to a previous communication (Fraser, 1957) for an outline of the general procedure in preparing the media used in this study.

Yolk dialysate. One hundred and fifty ml. of yolk from unincubated fertile eggs were dialyzed against 75 ml. of chick Ringer's solution in cylinders for two days at 5° C. The dialysate was then collected and stored under refrigeration until used.

Dialyzed yolk. One hundred ml. of whole yolk were dialyzed in the cold for three days against large volumes of the Ringer's solution. Dialysis proceeded in 10-liter flasks, the saline contents of which were changed on three occasions. The contents of the casings, representing the large molecule fraction of whole yolk, were then collected for use.

Boiled dialysate. Fifty ml. of freshly prepared yolk dialysate were gently refluxed for 10 minutes prior to use.

Ether partition of yolk dialysate. Fifty ml. of freshly prepared yolk dialysate were shaken on three occasions with 15 ml. of redistilled ethyl ether in a separatory funnel. The pooled ether phases were condensed at 45° C. under vacuum to near dryness. Five ml. of chick Ringer's were added and the remaining ether was distilled. Ether was removed from the aqueous phase in the same manner.

Acid hydrolysis of dialyzed yolk. The preparation of amino acids by the hydrolysis of dialyzed whole yolk used in the present study has been described by Block *et al.* (1958). Twenty ml. of dialyzed yolk were boiled under reflux with 40 ml. of 8 N sulfuric acid for 20 hours. To this, hot saturated barium hydroxide was added until a pH of 11 had been reached. After distillation *in vacuo* had removed the free ammonia, the excess barium was precipitated by an equivalent amount of 1 N H_2SO_4. The $BaSO_4$ was centrifuged off and the supernatant fluid reduced in volume to dryness. The residue was then taken up in 10 ml. of chick Ringer's solution for use.

Chromatographic Analysis of Yolk Dialysate

Twenty ml. of the yolk dialysate, prepared as indicated above, were reduced in volume to zero under mild heat from an infra-red lamp, and while agitated by a jet of air. The excess salt was removed by solvent extraction as outlined by Harris (1953). Drops of the concentrated aqueous phase, following ether extraction, were applied to Whatman No. 1 paper, measuring 18 by 22 inches. Two-

dimensional chromatograms were prepared by descending chromatography with water-saturated phenol as the first solvent and lutidine-collidine-water (3:1:1 by volume) as the second. After drying, the papers were sprayed with a 0.1% ninhydrin solution in 95% ethanol. On drying in an oven, the spots that appeared were compared with those on the amino acid map of Dent (1948). Similar chromatograms were made of the dialysate concentrate following oxidation with hydrogen peroxide, a procedure that is necessary for the identification of cysteine.

Figure 1 illustrates the identification of the free amino acids in yolk. In all there are thirteen such compounds readily distinguishable by this assay method. Spots numbered 1, 2 and 3 appeared only from a preparation following oxidation.

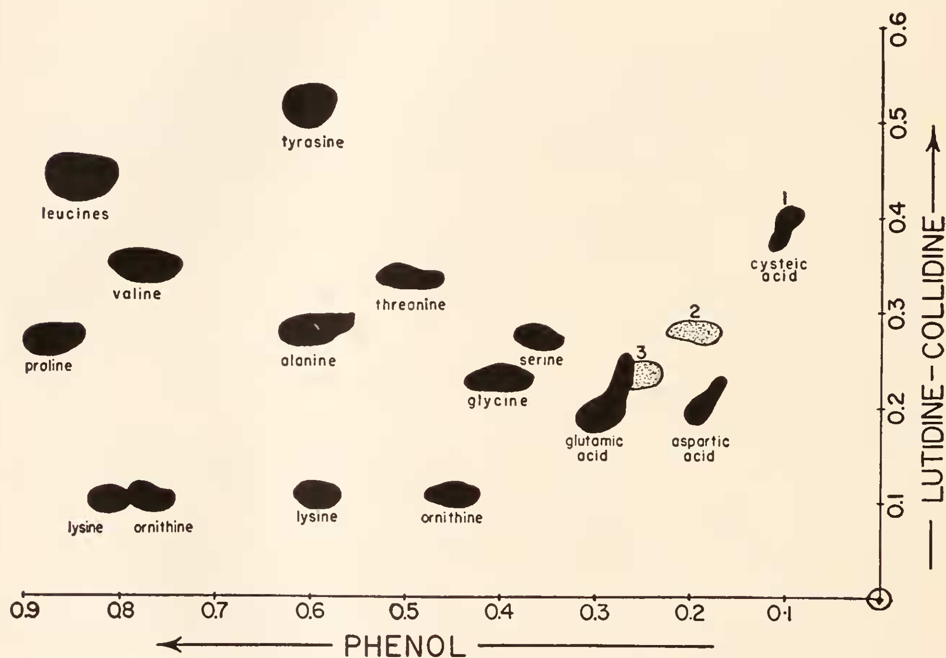

FIGURE 1. Two-dimensional chromatogram showing the amino acids detected free in yolk dialysate.

Grey colored spots indicated by numbers 2 and 3 do not coincide with any shown on Dent's map, and hence are unidentified at this time. The presence of cysteic acid, however, indicates the presence of free cysteine.

Barium and Alcohol Fractionation of Yolk and Albumin Dialysates

To establish more precisely the requirements of the young chick blastoderm from yolk and albumen in the formation of somites, a barium and alcohol fractionation of these food sources was undertaken. The procedure has been indicated in a general way by Harris (1953).

The pH of 15 ml. of yolk dialysate was brought to 8.2 by the careful addition

of 0.1 *M* NaOH. To this were added 2 ml. of 1 *M* $BaCl_2$, the pH of which had previously been adjusted to 8.2. The resulting mixture was placed in the freezing compartment of a refrigerator for 30 minutes and then centrifuged. The pellet was washed with five drops of $BaCl_2$ (pH 8.2) and then brought into solution with a small amount of doubly distilled water. To this was added a stoichiometric amount of 1 *M* Na_2SO_4 to precipitate all of the barium. The resulting supernatant was retained as the "*barium insoluble*" fraction.

The supernatant from this procedure was further fractionated by ethanol. The barium-soluble solution obtained was treated with sufficient molar sodium sulfate to precipitate all of the barium. The supernatant was reduced in volume to 10 ml. and chilled. To this were added 40 ml. of chilled absolute ethyl alcohol, thus bringing the concentration of the alcohol to 80 per cent. After further chilling for 30 minutes the preparation was centrifuged, and the pellet (the "*barium-soluble, alcohol-insoluble*" fraction) was washed with 5 drops of cold 80 per cent ethanol. The supernatant from this fractionation is termed the "*barium-soluble, alcohol-soluble*" fraction.

Table I

Assay of barium and alcohol fractions of yolk and albumen for nitrogen and phosphorus

Fraction	Yolk			Albumen		
	P (μg./ml.)	N (μg./ml.)	P/N	P (μg./ml.)	N (μg./ml.)	P/N
Barium-insoluble	104	10	10.40	3.2	8	0.40
Barium-soluble, alcohol-insoluble	10.8	9	1.20	12.5	26	0.48
Barium-soluble, alcohol-soluble	8.8	664	0.01	8.6	98	0.09
Whole dialysate	129	742	0.17	25.7	128	0.20

The three fractions thus obtained were placed individually in Stender dishes and the volume of each reduced to zero with mild heat and air agitation. Excess salt was removed and the volume again reduced to zero. To each were added 4 ml. of chick Ringer's solution and the resulting fractions set aside for incorporation into media.

A similar fractionation was carried out on albumen dialysate.

Assay of Barium and Alcohol Fractions for Phosphorus and Nitrogen Content

Umbreit *et al.* (1949) have listed the phosphorylated intermediates known to be present in the fractions derived from barium and alcohol treatment. In order to determine the relative concentrations of the various esterified compounds in the fractions used in the test media, an analysis was made on the fractions for nitrogen and phosphorus.

The results of such an analysis are shown in Table I. Total phosphorus was determined by the method given by Umbreit *et al.* (1949) after Fiske and Subbarow (1925). The nesslerization method of Koch and McMeekin (1924) for nitrogen was employed. Phosphorus and nitrogen determinations were made, as

indicated in the table, on the whole dialysate, the "barium-insoluble," "barium-soluble, alcohol-insoluble," and "barium-soluble, alcohol-soluble" fractions. Preparations from both yolk and egg white were assayed.

Contents of the Media

The contents of the media used in the present investigation are given in Table II, along with the amounts of each expressed in milliliters. Phenol red, in concentration of 0.01 per cent, was used as an inside pH indicator. Moreover, this dye also serves by coloring the medium, thus making it easier for the observer

Table II

Components of media used in the experiments and somite development in explanted chick embryos

Test material	Volume of test material	Ringer	Phenol red	Penicillin streptomycin	Phosphate buffer	Bicarbonate buffer	Total volume	No. embryos	Aver. no. pairs somites ± stand. error
None	—	33	2	2	2	1	40	25	0
Whole yolk	20*	23	2	2	2	1	50	28	7.0 ± 0.6
Yolk dialysate	20	13	2	2	2	1	40	56	7.4 ± 0.3
Dialyzed yolk	20*	23	2	2	2	1	50	42	0
Dialyzed yolk + glucose	28**	15	2	2	2	1	50	22	0
Boiled yolk dialysate	20	13	2	2	2	1	40	45	2.0 ± 0.5
Dialyzed yolk hydrolysate	2	1.3	0.2	0.2	0.2	0.1	4	30	0
Dialyzed yolk hydrolysate + glucose	2.8***	0.5	0.2	0.2	0.2	0.1	4	38	3.2 ± 0.5
Ether-soluble fraction	2	1.3	0.2	0.2	0.2	0.1	4	30	0
Saline-soluble fraction	20	13	2	2	2	1	40	32	7.9 ± 0.3
Free amino acids + glucose	17†	16	2	2	2	1	40	41	2.5 ± 0.3
Barium-insoluble fraction of yolk	3.5††	3.1	0.4	0.4	0.4	0.2	8	48	1.5 ± 0.2
Barium-soluble, alcohol-insoluble fraction of yolk	3.5	3.1	0.4	0.4	0.4	0.2	8	52	0.4 ± 0.2
Barium-soluble, alcohol-soluble fraction of yolk	3.5	3.1	0.4	0.4	0.4	0.2	8	40	1.8 ± 0.4
Barium-insoluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	38	0
Barium-soluble, alcohol-insoluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	45	2.2 ± 0.5
Barium-soluble, alcohol-soluble fraction of albumen	3.5	3.1	0.4	0.4	0.4	0.2	8	36	1.0 ± 0.3

* Twenty-five ml. of whole or dialyzed yolk were shaken with 50 ml. of Ringer's. Twenty ml. of the froth-free liquid were decanted for use.

** Eight ml. of 1% glucose plus 20 ml. of dialyzed albumen prepared as above.

*** Four-fifths ml. of 1% glucose plus 2 ml. of dialyzed yolk acid hydrolysate.

† One ml. of a 100 mg. % solution of each of the 13 amino acids found free in yolk dialysate plus 4 ml. of a 2% glucose preparation. Final concentrations: each amino acid, 2.5 mg. %; glucose, 200 mg. %.

†† The same amount (3.5 ml.) of the barium and alcohol fractions of both yolk and albumen was used.

to see the explanted embryos. The preparation of the penicillin-streptomycin solution used to prevent bacterial growth, and the phosphate and bicarbonate buffers has been described previously (Fraser, 1956). Throughout the period of culture the pH of the media remained near 7.6. The concentration of the agar used in preparing the semi-solid gels was 250 mg.%. The Ringer's solution was prepared in accordance with the formula given by Spratt (1947), with NaCl content reduced to 123 millimolar (Howard, 1953). In previous communications (Fraser, 1956, 1957) the details of preparing the media have been given. When the volume of the medium was small, 4 or 8 ml., it was poured into depression slides supported on cotton rings within petri dishes. The media of larger volume were placed in watch crystals held in similar fashion. The cotton rings supporting

the containers of media were moistened with sulfadiazine (0.25 per cent) as a further precaution against microbial contamination.

Results and Discussion

The development of somites in DPS explants cultured on the media outlined above for 22 hours is given in Table II.

One of the most striking observations is that the food materials used in this morphogenetic process are present free in the dialyzable (small molecule) fraction.

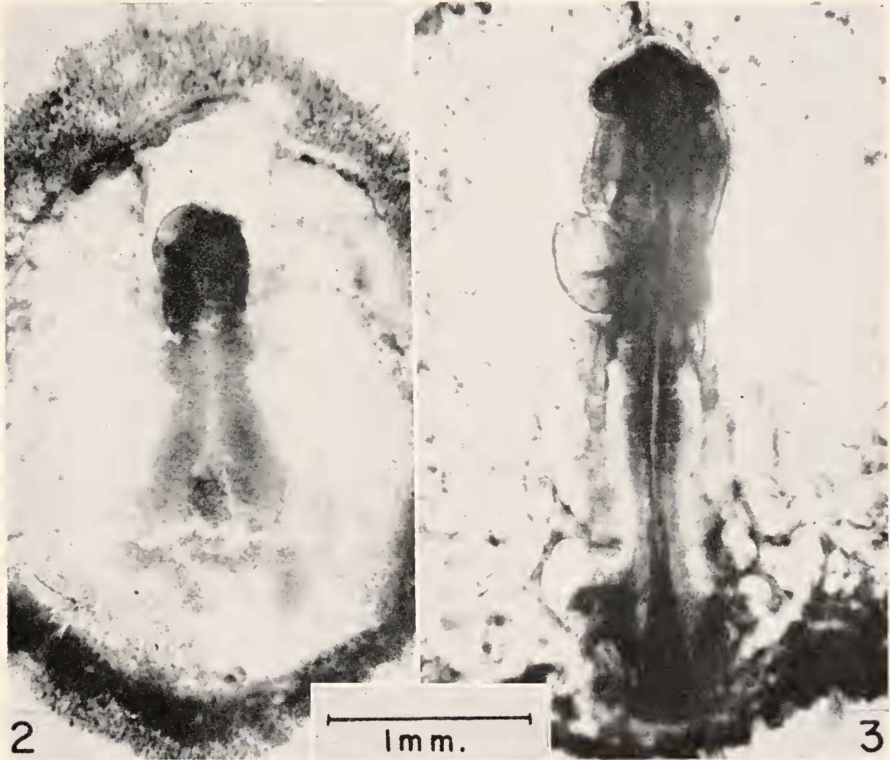

Figure 2. Photograph of a DPS blastoderm cultured for 22 hours on dialyzed whole yolk. Note absence of any appreciable development.

Figure 3. Photograph of an embryo of similar age cultured for 22 hours on a medium containing yolk dialysate. Note the formation of somites.

The same was found to be true when the egg white was assayed (Fraser, 1957). Somites developing in explants on the dialyzable fraction of whole yolk were equal in number to those in explants cultured on the yolk medium itself. On the other hand, no somites formed in blastoderms cultured on the large molecule moiety, even when glucose was added to the medium. This observation would indicate, in contrast to the conclusion of Taylor and Schechtman (1949), that the embryo in culture cannot use native protein alone from either yolk or albumen

for early morphogenesis. Figures 2 and 3 show these results. Blastoderms cultured on dialyzed yolk appear the same as those cultured on dialyzed albumen or on a saline-agar (non-nutrient) medium.

The chick embryo cultured *in vitro* can, however, use the protein moiety of yolk following hydrolysis of the polymers, when supplemented by glucose as an energy source. This observation would suggest that at this stage of development the embryo lacks sufficient proteolytic enzymes for its early morphogenic requirements, and relies almost entirely (if not completely) on molecules of simpler form found free in its environment.

There is, as yet, no complete characterization of the small molecule nutrients used by the chick blastoderm for somite development. Ether fractionation reveals that all materials used are in the aqueous phase. The inability of the very early embryo to use fat as a substrate for development has been noted previously (Fraser, 1956, 1957). It is equally true that some of the somite-forming capacity of yolk dialysate is curtailed by heat. A similar heat-labile fraction has been found in albumen dialysate (Fraser, 1957). There is further support for the presence of a heat-labile component in the observation that somite development is somewhat depressed in explants cultured on a medium containing all of the amino acids found free in yolk.

As one would expect, the bulk of nitrogen, signifying the presence primarily of amino acids, is found in the barium- and alcohol-soluble fraction of both yolk and albumen. In view of the fact that there is some development of somites in explants cultured on the amino acids detected in this portion of the avian egg, it is not surprising that this fraction derived from barium and alcohol precipitation should yield a similar result. This is borne out by observation.

There has been, however, some dispute as to whether the early chick blastoderm can utilize phosphorylated carbohydrate intermediates for its development. Needham and Nowinski (1937) have come to the conclusion that such substances cannot serve as nutrients for the early chick embryo. Novikoff, Potter and Le Page (1948), on the other hand, have demonstrated the presence of phosphorylated intermediates in embryonic chick homogenate. Fraser (1956) has reported the presence of cytochrome oxidase in blastoderms as young as the beginning streak stage. The great importance of glucose in the development of the early chick embryo has been repeatedly demonstrated (Spratt, 1949; Fraser, 1954). Moreover, unpublished work from this laboratory has indicated that only a small portion of carbohydrate metabolism in the young avian embryo is directed through the phosphogluconate shunt. It is evident, therefore, that unless some other, much less likely, mechanism for sugar oxidation exists, carbohydrate must be handled in the conventional phosphorylated manner. If this assumption is true, we might well expect that phosphorylated intermediates, when offered to the embryos, would be metabolized. This contention is borne out by observation that somite development proceeds to a limited degree in explants cultured on media containing these carbohydrate phosphate esters.

Umbreit *et al.* (1949) have listed the various phosphorylated compounds precipitated by barium and alcohol fractionation. According to them, most of these intermediates in carbohydrate metabolism are brought down in the barium-soluble, alcohol-insoluble fraction. Comparison of P/N ratios would indicate that such

fractionation precipitates different compounds in albumen than in yolk. The wide discrepancies in phosphate values point to this conclusion. Differences in somite counts in embryos cultured on these fractions add further support. Finally, embryos were cultured on media containing the barium-soluble, alcohol-insoluble fractions of both yolk and albumen dialysates which had been boiled. No somites formed in blastoderms explanted on either of these media. There was no suppressing effect, however, by heat on the barium-insoluble fraction of yolk. We might conclude from this, then, that insofar as the development of somites is concerned, the explanted chick embryo utilizes at least one different component which is present in yolk but not in egg white.

Finally, we should attempt to answer the provocative question why it is that less than the maximal number of somites form in embryos cultured on certain media. Actually the total number of somite pairs found in explants cultured under optimal conditions on any yolk or albumen medium is somewhat less than that seen in embryos grown *in ovo* for a similar period of time. We can therefore conclude that the *in vitro* culture technique falls short in providing the embryo with appropriate environmental conditions other than of a nutritive nature. But insofar as somite development in explanted embryos alone is concerned, such differences must have a nutritional basis, since physical environmental conditions are presumably equivalent.

The experiments in this and the former paper in this series (Fraser, 1957) have shown that the formation of somites in the chick embryo cultured *in vitro* is dependent on a number of chemical constituents in the food supply. We might therefore profitably think that a submaximal number of somites in explants results when one or more of the nutritional requirements for the maximal formation is lacking. This deficiency may then act to limit the number of somites which could develop within a specified period of time. It is only when all of the components needed for this morphogenetic event are present that the maximum number of somites will form.

Summary

1. Definitive primitive streak chick embryos have been cultured *in vitro* on media containing various fractions of whole yolk. Other embryos were grown on agar gels containing fractions derived by barium and alcohol fractionation of yolk and albumen dialysates. Following 22 hours of incubation, the blastoderms were mounted and examined for the development of somites.

2. The nutritional components of whole yolk are all present in the saline-soluble, dialyzable moiety, although the embryo can use the acid hydrolysate of dialyzed yolk, when accompanied by glucose, for this morphogenetic process.

3. The results, derived from an assay of barium and alcohol fractionation of both yolk and egg white, indicate that the early chick embryo can use, to limited extent, certain phosphate esters of carbohydrates in the formation of somites. There appear to be different phosphorylated materials in yolk than in albumen used by the embryo.

4. Insofar as whole yolk utilization is concerned for the formation of somites, there is evidence that the chick embryo grown *in vitro* uses: (1) glucose, (2) the amino acids found free in yolk, (3) a heat-labile, uncharacterized factor, and (4)

certain phosphorylated carbohydrate intermediates. It may also use, but to a very limited extent, the products of proteolysis of yolk proteins.

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